

Indole-2-carboxamide Inhibitors of Human Liver Glycogen Phosphorylase[†]

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Type 2 diabetes (formerly non-insulin-dependent diabetes mellitus) is a severe and prevalent disease. Diabetics may suffer debilitating eye, kidney, and nerve damage and are at risk of premature death due to these and other diabetic complications.¹ Tight control of plasma glucose, achieved by intensive insulin therapy, reduces the incidence and progression of diabetic complications in type 1 and type 2 diabetics.^{2,3} Unfortunately, achieving control is often impossible with current oral hypoglycemic agents. Moreover, their chronic use often brings, in addition to toleration, fear of dangerous hypoglycemic episodes. Safer and more effective therapy is urgently needed.

We targeted liver glycogen phosphorylase (EC 2.4.1.1) as an approach to controlling elevated glucose levels. The liver is the predominant source of plasma glucose in fasting type 2 diabetics.⁴⁻⁷ Figure 1 shows, schematically, selected details of hepatic glucose synthesis, storage, and release.⁸ Glycogen phosphorylase^{9,10} releases glucose-1-phosphate from glycogen. Although significant evidence exists of an important role for glycogenolysis in hepatic glucose production,¹¹⁻¹⁴ glycogen phosphorylase is not a widely pursued antidiabetic target, partly because of conclusions that the high glucose levels in type 2 diabetics arise independently from elevated gluconeogenesis.^{7,15,16} Recent data, however, showing that much of glucose arising from gluconeogenesis has cycled through glycogen,¹⁷ exposes a possibility that inhibition of glycogen phosphorylase could suppress hepatic glucose arising from both pathways.¹⁸ Orally active glycogen phosphorylase inhibitors¹⁹ would clearly be valuable additions to this research area.

Activity of liver glycogen phosphorylase is regulated physiologically by hormonal and neuronal signals which alter phosphorylation at serine 14. Only the phosphorylated enzyme has significant activity. The liver isozyme is also regulated by ligands which bind to a distant allosteric site, termed the nucleotide activation site. AMP binds here as an activator,²⁰ while ATP and

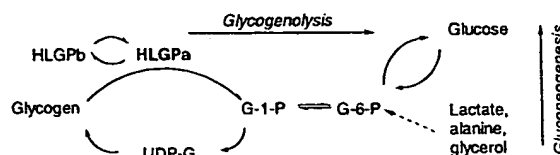


Figure 1. Selected details of hepatic glycogenolysis and gluconeogenesis. Abbreviations: HLGPa, human liver glycogen phosphorylase *a* (phosphorylated, active form); HLGPb, unphosphorylated form; UDP-G, uridine diphosphoglucose; G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate.

glucose-6-phosphate (G-6-P) bind here as inhibitors.²¹ Synthetic G-6-P analogues²² and a dihydropyridinedicarboxylic acid²³ also inhibit at the nucleotide activation site of rabbit muscle glycogen phosphorylase *b* (RMGPb). Most reported phosphorylase inhibitors¹⁰ are glucose or other sugar-based analogues,²⁴ which have in numerous instances been shown to bind at the catalytic site of RMGPb.²⁵⁻²⁹ Caffeine and other nucleosides inhibit at another allosteric site, termed the purine inhibitor site.³⁰ No physiologic role or ligand for the purine inhibitor site is known, although xanthines may regulate phosphorylase activity here in certain physiologic states.^{31,32} Inhibition by caffeine and glucose is synergistic, because of cooperative ordering of both binding sites.²⁵ This is an attractive property, for a therapeutic inhibitor which would lose potency as glucose levels fall should offer reduced risk of severe hypoglycemia.

Recently, we reported finding the orally active glycogen phosphorylase inhibitor **1** (Table 1) by high-throughput screening against recombinant human liver glycogen phosphorylase *a* (rHLGPa).¹⁸ Other inhibitors of the human liver isoform³³ of glycogen phosphorylase have not been described. Here, we demonstrate that compounds in two series derived from **1** (Tables 1 and 2) are also potent inhibitors of rHLGPa and have cellular and oral activity. Their synthesis and characterization are also described.

The simple, heterocyclic, and asymmetric structure of **1** was exciting, as was the opportunity to explore the importance of these features to its high inhibitory activity. Inhibitor **1** and other (3*S*,2*R*)-2-amino-3-hydroxy-4-phenylbutyric acid derivatives were prepared as shown in Scheme 1. Methyl ester **28** was formed on treatment of cyanohydrin **27**³⁴ with anhydrous HCl in methanol and purified by extraction and recrystallization. Carbodiimide-mediated coupling with commercially available indole-2-carboxylic acids gave methyl ester **10** and the fluoro, bromo, and deschloro analogues **29-31** in high yield. Some of these products contained up to 10% of the diester resulting from hydroxyl acylation, and were hydrolyzed with aqueous sodium hydroxide in methanol, giving acids **11** and **32-34** in pure form after extraction and trituration. Coupling with dimethylamine and methylamine hydrochlorides gave target amides **1** and **5-8** in good yields. The stereoisomers of **1** (**2-4**) were also synthesized this way, from the stereoisomers of methyl ester **28**, which were obtained as their hydrochlorides in quantitative yield by refluxing the commercial (2*S*,3*R*)-, (2*R*,3*R*)-, and

[†] This manuscript is dedicated to the occasion of Professor E. J. Corey's 70th birthday.

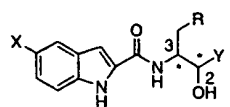
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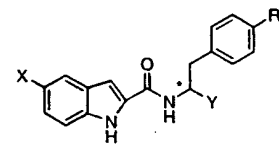
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Table 1. Structures and in Vitro Phosphorylase Inhibitory Activity of 3-[(Indole-2-carbonyl)amino]-2-hydroxy-4-phenylbutyric Acid Analogues


compd	X	R	Y	*	rHLGPa IC ₅₀ , nM ^a	cell IC ₅₀ , μM ^b
1	Cl	Ph	CONMe ₂	3S,2R	110 ± 12	1.5
2	Cl	Ph	CONMe ₂	3R,2S	1800 ± 320	
3	Cl	Ph	CONMe ₂	3R,2R	1700 ± 190	
4	Cl	Ph	CONMe ₂	3S,2S	8700 ± 1700	
5	F	Ph	CONMe ₂	3S,2R	170 ± 16	6.2
6	Br	Ph	CONMe ₂	3S,2R	97 ± 11	3.3
7	H	Ph	CONMe ₂	3S,2R	440 ± 48	>30
8	Cl	Ph	CONHMe	3S,2R	680 ± 37	>30
9	Cl	Ph	CONH ₂	3S,2R	1700 ± 140	
10	Cl	Ph	COOMe	3S,2R	210 ± 40	13
11	Cl	Ph	COOH	3S,2R	330 ± 31	>30
12	Cl	Ph	CH ₂ OH	3S,2R	6800 ± 1700	
13	Cl	Cy ^c	CONMe ₂	3S,2R	6500 ± 610	

^a Arithmetic mean ± SEM ($n \geq 3$, each in triplicate). ^b Inhibition of forskolin-stimulated glycogenolysis in SK-HEP-1 cells (pooled data from $n \geq 2$, each in triplicate). ^c Cyclohexyl.

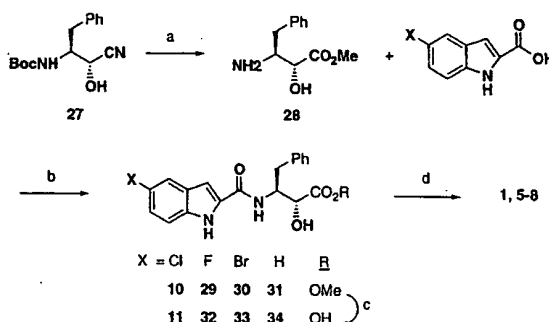
Table 2. Structures and in Vitro Phosphorylase Inhibitory Activity of *N*-(Indole-2-carbonyl) Phenylalanine Analogues


compd	X	R	Y	*	rHLGPa IC ₅₀ , nM ^a	cell IC ₅₀ , μM ^b
14	Cl	H	CONMe ₂	S	82 ± 10	1.9
15	Cl	H	CONMe ₂	R	260 ± 33	12
16	Cl	H	CONHMe	S	110 ± 10	5.2
17	Cl	H	CONHMe	R	220 ± 32	12
18	Br	H	CONMe ₂	S	110 ± 8	1.4
19	F	H	CONMe ₂	S	430 ± 44	2.3
20	H	H	CONMe ₂	S	400 ± 45	7.8
21	OMe	H	CONMe ₂	S	4700 ± 300	
22	Cl	H	CO(1-piperidin-4-yl)	S	260 ± 17	5.1
23	Cl	F	CO(1-piperidin-4-yl)	S	205 ± 16	1.9
24	Cl	H	COOMe	S	140 ± 18	>30
25	Cl	H	COOH	S	1700 ± 490	>30
26	Cl	H	CH ₂ OH	S	>10000 ^c	

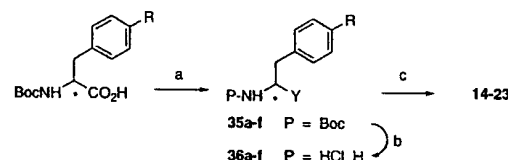
^a Arithmetic mean ± SEM ($n \geq 3$, each in triplicate). ^b Inhibition of forskolin-stimulated glycogenolysis in SK-HEP-1 cells (pooled data from $n \geq 2$, each in triplicate). ^c 23% inhibition at 10 μM.

(2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acids with chlorotrimethylsilane in methanol.³⁵ Less than 1% of **1** was present in isomers **2–4** by chiral stationary phase HPLC analysis. Primary amide **9** was obtained by heating methyl ester **10** with ammonia in methanol. Diborane reduction of acid **11** gave diol **12**. Cyclohexane **13** was prepared by steps b–d of Scheme 1, starting from (2*R*,3*S*)-3-amino-4-cyclohexylmethyl-2-hydroxybutyric acid isopropyl ester.³⁶

Phenylalanine-containing analogues of **1** were also synthesized (Scheme 2), by stepwise coupling from the C-terminus using *t*-Boc-protected phenylalanines. Methyl ester **24** and alcohol **26** were obtained from *L*-phenylalanine methyl ester hydrochloride and *L*-phenylalaninol, respectively. Hydrolysis of **24** with aqueous lithium hydroxide in THF gave acid **25**. *N*-Methylindole

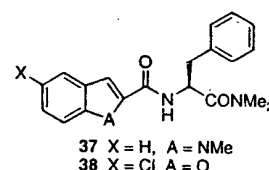
Scheme 1^a

^a Key: (a) HCl–MeOH, 60 h, 23 °C, then NaHCO₃/H₂O, 77%; (b) 1.0 equiv of 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride, 1.5 equiv of 1-hydroxybenzotriazole, dichloromethane, 23 °C, 18 h; (c) 2.0 equiv of NaOH, MeOH, H₂O, 25 °C, 2 h; (d), as for (b), but using also 1.1 equiv of either Me₂NH·HCl or MeNH₂·HCl and 1.1 equiv of triethylamine in DMF, 18 h, 23 °C.

Scheme 2^a

^a R, Y, and X are defined in Table 2. Key: (a) 1.0–1.1 equiv of dimethylamine, methylamine, or 4-hydroxy-1-piperidine hydrochloride, 1.1 equiv of Et₃N, 1.0 equiv of DEC, 1.5 equiv of HOBT, CH₂Cl₂, 0–20 °C, 18 h; (b) HCl–dioxane, 23 °C, 1 h; (c) 1.0 *X*-indole-2-COOH, 1.0 DEC, 1.5 HOBT, 1.0 Et₃N, CH₂Cl₂, 23 °C, 18 h.

37 and benzofuran **38** were obtained from (*S*)-phenylalanine dimethylamide hydrochloride (**35a**) and 1-methylindole-2-carboxylic acid or 5-chlorobenzofuran-2-carboxylic acid.



Compounds were assessed for ability to inhibit rHLGPa-catalyzed release of phosphate from glucose-1-phosphate (reverse direction of the physiological reaction) in the presence of glycogen and a high physiological concentration of glucose (7.5 mM).¹⁸ The (3*S*,2*R*) stereochemistry of **1** is optimal as seen in the reduced inhibitory activity of stereoisomers **2–4** (Table 1). Inhibitory activity was also sensitive to changes in the indole 5-substituent. Activity was completely retained in bromo analogue **6**, but diminished 1.5- and 4-fold in the 5-fluoro and deschloro analogues (**5** and **7**, respectively). C-Terminally modified analogues showed considerable variation in their inhibitory activity. Monomethylamide **8** and primary amide **9** showed 6- and 15-fold lower activity than **1**. The synthetic intermediates **10** (methyl ester) and **11** (carboxylic acid) were closer in activity to **1**. Diol **12** showed about a 60-fold reduction of inhibitory activity relative to **1**, as did the cyclohexylmethyl side chain analogue **13**.

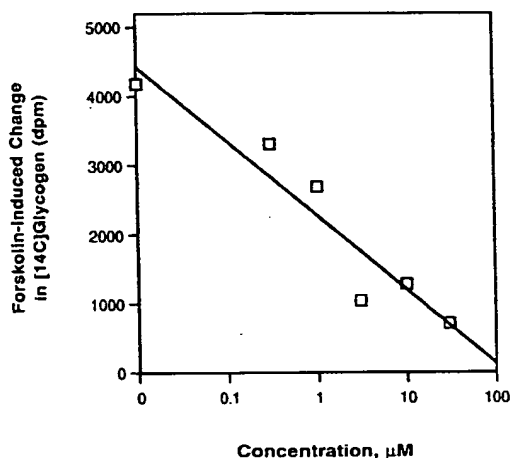


Figure 2. Difference in ^{14}C glycogen content (dpm) of [^{14}C]glycogen prelabeled, vehicle-treated human SK-HEP-1 cells vs those treated with 50 μM forskolin (4 h incubations), at different concentrations of **1**. Mean of 3–5 experiments, each in triplicate.

Phenylalanine derivatives also have high rHLGPa inhibitory activity (Table 2). L-Phenylalanine dimethylamide **14** retained the potency of **1**, inhibiting rHLGPa with an IC_{50} of 82 nM. In contrast to the trend observed for stereoisomers of **1**, D-phenylalanine **15** (260 nM) was only about 3-fold less active than its enantiomer **14**. Similar moderate dependence of rHLGPa inhibitory activity on absolute configuration also prevailed between the enantiomeric monomethylamides **16** and **17**. Potency was retained in methyl ester **24** (140 nM) and diminished in acid **25** (1700 nM) and in the corresponding alcohol **26** (9300 nM). Changing the indole 5-substituent in the phenylalanines reduced inhibitory activity in the order $\text{Cl} \approx \text{Br} > \text{F} \approx \text{H} > \text{OCH}_3$ (analogues **14**, **18**–**21**, respectively). Thus, in the β -amino- α -hydroxybutyric acid and phenylalanine series, potency was affected very differently by changes in stereochemistry and C-terminal amide functionality, but similarly by changes to the indole 5-substituent. These results suggest that compounds in both series inhibit rHLGPa at the same site, with the 1*H*-indole-2-carboxamide moiety making a significant contribution to binding affinity. Sharply reduced activity of *N*-methylindole **37** and benzofuran **38** (rHLGPa $\text{IC}_{50} > 10 \mu\text{M}$) is consistent with this hypothesis. Structural and kinetic experiments are being performed to understand the inhibition mechanisms of these compounds.

To evaluate the activity of inhibitors in cells, an assay was established in a human liver-derived tissue culture cell line. In the absence of inhibitor, addition of forskolin to SK-HEP-1 cells activated glycogen phosphorylase (phosphorylation of HLGPa to HLGPb), resulting in degradation of ^{14}C -labeled glycogen (Figure 2). Inhibitor **1** blocked the forskolin effect with an IC_{50} of 1.5 μM (concentration-dependent), a value similar to that previously obtained in primary cultures of isolated human hepatocytes ($\text{IC}_{50} = 2.1 \mu\text{M}$).¹⁸ Likewise, inhibitor **23** showed IC_{50} values of 1.9 μM (SK-HEP-1, Table 2) and 2.7 μM (human hepatocytes). Other analogues of **1** in both series are also potent inhibitors of glycogenolysis in SK-HEP-1 cells (Tables 1 and 2). These data indicate that **1** and analogues readily penetrate

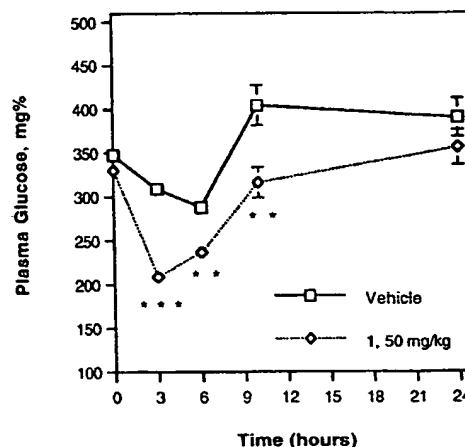


Figure 3. Time course of glucose lowering following oral dosing of **1** in diabetic *ob/ob* mice. Vertical lines show SEM. Each point represents 35–66 mice per group. Significance ($***P < 0.001$, $**P < 0.01$) by unpaired *t*-test relative to vehicle control groups. Similar results were obtained in an independent experiment of the same design.

Table 3. Hypoglycemic Responses Resulting from Oral Dosing of Glycogen Phosphorylase Inhibitors in Diabetic *ob/ob* Mice

compd	doses inactive, mg/kg po	dose active, ^a mg/kg po	% glucose lowering at active doses ^b	significance (<i>P</i> value)
1	15, 10	50 ¹⁸ 25 ¹⁸	40 32	0.05 0.05
4	50			
5	10	25	33	0.05
6	50			
10	50			
16	25	50	49	0.01
17	25	50	39	0.05
18	50			
19	50			
20	25	50	40	0.01
22	10	25	36	0.05
23	5	50	44	0.001
		25	45	0.001
		15	26	0.01
		10	24	0.05
24	50			

^a Significant ($P \leq 0.05$) plasma glucose lowering at 3 h postdose, relative to vehicle treated controls ($n = 10$ per group), in drug treated *ob/ob* mice. Statistical analysis by two-tailed, unpaired Student *t*-test. ^b Percent decrease in drug-treated plasma glucose concentration, relative to the vehicle-treated control within the experiment.

liver cells. Cell potency often correlated with potency in the purified enzyme assay (e.g., the two pairs of phenylalanine enantiomers **14**–**17**). Certain potent inhibitors, however, were less effective inhibitors of glycogenolysis in cells (cf. **7**, **8**, **11**, **24**). These data do not reveal factors responsible for these differences.

Oral administration of **1** to diabetic (*ob/ob*) mice in the fed state at 50 or 25 mg/kg lowers plasma glucose 3 h later.¹⁸ The time course of this effect was determined using a 50 mg/kg po dose of **1** (Figure 3). Glucose lowering was statistically significant at 3, 6, and 10 h, but not at 24 h, with the largest drop occurring at 3 h. Analogues of **1** were screened for oral activity at this timepoint (Table 3). The (2*S*) stereoisomer of **1** (**4**, not a potent rHLGPa inhibitor) did not show oral hypoglycemic activity at 50 mg/kg, which is consistent with phosphorylase inhibition being the mechanism of the

hypoglycemic action of **1** in vivo. Like **1**, fluoro analogue **5** also showed oral activity at 25 mg/kg, but not at 10 mg/kg. Bromoindole **6** and the methyl ester **10**, both potent inhibitors in vitro, did not show oral activity at 50 mg/kg. Several phenylalanines also showed oral activity. Hydroxypiperidines **22** and **23** were active at 25 mg/kg, and unlike **1**, **23** produced marked glucose lowering at 10 mg/kg. Furthermore, under these same conditions **23** did not alter plasma insulin levels (222 ± 26 microunits/mL, vehicle-treated versus 234 ± 32 microunits/mL, **23**-treated). Based on this finding, coupled with our previous observation that **1** did not affect insulin secretion from isolated rat islets,¹⁸ we conclude that the observed glucose-lowering appears to be directly due to hepatic glycogenolysis inhibition and cannot be attributed to a mild insulin secretory activity. Additional experiments are being performed to better understand the origin of in vivo potency differences in these series.

Important questions arise when this approach is considered for antidiabetic therapy. Two are whether the inhibitor effects can be confined to the liver and whether glycogen will accumulate to unacceptably high levels as in glycogen storage diseases. Experiments to address these questions have given promising results. IC₅₀ values for inhibition of the human muscle isoform of glycogen phosphorylase (rHMGP_a)³⁷ were 200 ± 7 nM (**1**), 180 ± 22 nM (**14**), and 430 ± 15 nM (**15**), which are 2–3-fold higher than for rHLGP_a. Additionally, compound **23** is a potent inhibitor of the muscle enzyme (83 ± 3 nM), but did not substantially impair glycogen mobilization in muscle, in situ, under conditions where liver glycogenolysis should be inhibited.³⁸ The absence of a muscle effect may be a result of low inhibitor concentration in muscle. Another question is whether glucose lowering can be sustained chronically by this mechanism. To this end, we have observed that **23** produces glucose lowering in diabetic *ob/ob* mice after daily b.i.d. dosing for up to 2 weeks.³⁸

In summary, two series of indole-2-carboxamides derived from **1** contain potent inhibitors of human liver glycogen phosphorylase which are active in cells, and produce hypoglycemic activity on oral administration in a rodent model of type 2 diabetes. Compound **23** (CP-320626) produced oral activity at 10 mg/kg. These compounds should enable further characterization of the role of glycogenolysis in normal and disease states, and determination of the potential of glycogen phosphorylase as a therapeutic target in type 2 diabetes.

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Supporting Information Available: Synthetic procedures and characterization data for all compounds, chiral HPLC method, and biological procedures (28 pages). Ordering information is given on any current masthead page.

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